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Purification and characterization of a phospholipase A_2 from human ileal mucosa

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We purified a phospholipase A_2 (PLA_2) from human ileal mucosa to homogeneity. Its NH_2-terminal amino acid sequence, amino acid composition, molecular weight, and elution behavior on reverse phase high-performance liquid chromatography were identical to those of human group II PLA_2 purified from synovial fluid or spleen. The ileal PLA_2 preferred anionic phosphatidylglycerol as substrate. On immunoblot analysis, human ileal mucosa gave more intense immunoreactivity with anti-human synovial fluid PLA_2 antibody, at the same position as the purified enzyme, than the cecal mucosa. Northern blot analysis also showed that the level of group II PLA_2 mRNA in the ileal mucosa was greater than that in the cecal mucosa. The enzyme was rather uniformly distributed over the colonic mucosa, from cecum to sigmoid colon. These results indicate that the ileal mucosa contains group II PLA_2, and that its expression in the ileal mucosa was higher than that in the colonic mucosa.

Introduction

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the fatty acyl ester bond at the *sn*-2 position of glycero-3-phospholipids. This enzyme is thought to participate in regulating phospholipid metabolism in biomembranes and eicosanoid biosynthesis [1]. The enzyme exists in almost every type of cell studied so far [2]. Recently, it has been demonstrated that calcium-dependent PLA_2s of mammalian origin can be classified into at least three groups according to their distinct characteristics in primary structure [3–7]: the pancreatic type (group I), the viperid and crotalid type (group II), and the cytosolic high molecular weight type.

Recent studies suggest that calcium-dependent PLA_2s participate in the inflammatory response either through a direct action or through an indirect action via its metabolites [8–11]. As for the intestine, arachidonate-derived chemical mediators may be involved in the pathogenesis of chronic inflammation. Raised intestinal contents of leukotriene B_4 and prostaglandins were described in inflammatory bowel disease [12–14]. In this context, increased PLA_2 activity was reported in ileal and colonic mucosa of patients with Crohn's disease [15,16]. Very recently, we reported that both PLA_2 activity and immunoreactive group II PLA_2 levels were elevated in sera of patients with active inflammatory bowel disease [17,18]. However, PLA_2 in human ileal and colonic mucosa has not yet been characterized in detail.

In the present study, we have purified a PLA_2 from human ileal mucosa to homogeneity, and determined its molecular properties including its NH_2-terminal amino acid sequence. In addition, we have performed immunochemical and molecular biological analyses on the distribution of PLA_2 in human lower gastrointestinal tract using histologically normal ileal and cecal mucosa surgically obtained from a patient with Crohn's disease, and biopsy specimens of histologically normal ileal and colonic mucosa obtained through colonoscopy.

Materials and Methods

Materials

Human terminal ileums were supplied from autopsy materials, and stored at −35°C until use. Surgical specimens of histologically normal ileal and cecal mucosa were obtained from a patient with Crohn's disease

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Abbreviations: $C_{12}E_8$, octaethylene glycol dodecyl ether; HPLC, high-performance liquid chromatography; PLA_2, phospholipase A_2; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

and stored at −80°C until use. Eighteen biopsy specimens of histologically normal mucosa were obtained from terminal ileum, cecum, ascending, transverse, descending, and sigmoid colon of 12 patients (8 men and 4 women) aged 24–64 years (mean age of 42.6) who underwent colonoscopy for check-up after endoscopic polypectomy, or investigation of gastrointestinal symptoms, or as a supplement of radiographic investigation for irregular bowel habit. One colonic polyp was found in 4 of them, and no abnormalities were found in the others. Informed consent was obtained in each case. These biopsy specimens were washed twice by cold saline and stored at −35°C until use.

S-Sepharose was obtained from Pharmacia. Trifluoroacetic acid (TFA), acetonitrile of high-performance liquid chromatography (HPLC) grade, and octaethylene glycol dodecyl ether ($C_{12}E_8$) were purchased from Nacalai Tesq. (Kyoto, Japan). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids. Other reagents were of analytical grade.

Human pancreatic PLA_2 was purified from human pancreatic juice as reported by Ono et al. [19] with some modifications. Human group II PLA_2 was purified from rheumatoid arthritic synovial fluid as reported [20]. The polyclonal antibodies directed against human pancreatic PLA_2 and synovial fluid PLA_2 were prepared by the same method as reported [21].

Assay for PLA_2 activity

PLA_2 activity was determined as described previously [20,22] using mixed micelles of 0.8 mM POPG and 5 mM cholate as substrate. Free fatty acids released by the PLA_2 action were labeled with 9-anthryldiazomethane, and the derivatized fatty acids were separated, and the oleic acid was quantitated by reverse phase HPLC using marganic acid as an internal standard. Calcium-dependent PLA_2 activity was estimated as the difference between the activity assayed in the presence of 5 mM $CaCl_2$ and that in the presence of 10 mM EDTA.

Purification of PLA_2 from human ileal mucosa

Human ileal mucosa was scraped off from the muscularis propia, and the scrapings (10 g wet weight) were homogenized in 100 ml of 10 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 105 000 × *g* for 60 min at 4°C. The pellet was homogenized in 10 mM Tris-HCl (pH 7.4) containing 1 M KBr. The homogenate was kept on ice for 60 min at 4°C to extract PLA_2 activity [20]. The homogenate was centrifuged at 105 000 × *g* for 60 min at 4°C. The supernatant was diluted 10-fold with 10 mM Tris-HCl (pH 7.4), and applied to a S-Sepharose column (6 × 3 cm) preequilibrated with 10 mM Tris-HCl (pH 7.4). The column was washed out with 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, and then PLA_2 activity was eluted in a stepwise manner with 10 mM Tris-HCl (pH 7.4) containing 1 M NaCl and 0.1% Triton X-100. The eluent was diluted with 10 mM Tris-HCl buffer (pH 7.4) to reduce the NaCl concentration to less than 0.1 M. The resultant solution was applied to a S-Sepharose column (2 × 3 cm) preequilibrated with 10 mM Tris-HCl (pH 7.4) containing 0.1% Triton X-100. After the column was washed with the same buffer, its outlet was connected to the bottom inlet of a Cellulofine GCL-300 m column (2 × 50 cm) preequilibrated with 10 mM Tris-HCl (pH 7.4) containing 0.3 M NaCl and 0.1% Triton X-100. Elution was performed with 10 mM Tris-HCl (pH 7.4) containing 1 M NaCl and 0.1% Triton X-100, and the concentrated PLA_2 solution was transferred to the gel filtration column. Then, the PLA_2 activity was chromatographed in an upward direction on the gel filtration column. The flow rate was 15 ml/h, and the volume of each fraction was 5 ml. The pooled PLA_2 active fractions were diluted 3-fold with 10 mM Tris-HCl (pH 7.4), and then the diluted solution was applied to a S-Sepharose column (10 × 5 mm) preequilibrated with 10 mM Tris-HCl (pH 7.4) to concentrate the PLA_2 and to replace Triton X-100 by $C_{12}E_8$ in the enzyme solution. The column was washed with 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 0.1% $C_{12}E_8$. The PLA_2 was eluted with 10 mM Tris-HCl (pH 7.4) containing 1 M NaCl and 0.1% $C_{12}E_8$. An aliquot (2.5 ml) of the resultant solution was subjected to a Cosmosil 5C8-300 reverse phase HPLC column (2.1 × 100 mm) preequilibrated with 0.1% TFA in water. Elution was performed with the linear gradient of acetonitrile in 0.1% TFA: 0–19% in 5 min, 19–38% in 45 min, and then 38–95% in 5 min at the flow rate of 0.1 ml/min. HPLC was performed with two Gilson Model 302 liquid delivery modules and a Gilson 811 dynamic mixer equipped with a 65-μl mixing chamber. The system was operated in the microflow mode and the effluents was monitored with a Gilson 116 detector equipped with an 1.6-μl microflow cell.

Immunoblot analysis

The surgical specimens of normal ileal and cecal mucosa were homogenized in 3 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA and 2 mM $MgCl_2$. Each sample containing 10 μg protein was electrophoresed on a 14% sodium dodecyl sulfate (SDS)-polyacrylamide gel under non-reducing conditions, and immunoblot analysis was performed as described previously [17].

RNA extraction and Northern blot analysis

Total RNA was extracted from the ileal and cecal

mucosa by the acid guanidium-phenol-chloroform method [23]. 10 μg of total RNA from each material was electrophoresed on a 1% agarose gel and hybridized with a radioactive human ileal group II PLA_2 cDNA probe as described [24]. The cDNA encoding the ileal PLA_2 was cloned by the essentially same method as reported [5]. The 830-kb insert subcloned into a plasmid vector was obtained by the digestion with *Xho*I and *Hin*dIII and purified by electrophoresis on low melting-point agarose gel. The probe cDNA was labeled with [α-^{32}P]dCTP using a multiprime-labeling kit (Amersham).

NH_2-terminal amino acid sequence and amino acid analysis

NH_2-terminal amino acid sequence analysis was performed by an Applied Biosystem 477A and a 120A PTH analyzer, and amino acid analysis was performed as reported [4].

Preparations of tissue extracts

Biopsy specimens of histologically normal ileal and colonic mucosa were homogenized with 30 vol. of 10 mM Tris-HCl (pH 7.4), and the homogenates were used for the assay of PLA_2 activity. An aliquot of the homogenate containing 100 μg protein was mixed with an equal volume of 10 mM Tris-HCl (pH 7.4) containing 2 M KBr. After the mixture was kept on ice for 1 h, it was centrifuged at 40000 $\times g$ for 40 min at 4°C. The supernatant was used for the assay of immunoreactive group II PLA_2 (IR-PLA_2 II), because the PLA_2 enriched in particulate fractions could be efficiently solubilized by this method [4]. IR-PLA_2 II content was determined by a sensitive radioimmunoassay specific to human group II PLA_2, using monoclonal antibodies raised against human splenic group II PLA_2 [25]. The inclusion of tissue extract containing 1 M KBr in the assay mixtures did not affect both the PLA_2 activity and the immunoreactivity with the antibodies.

Protein concentration determination

The protein concentrations of crude samples during the purification and surgical specimens of the normal ileal and colonic mucosa, and those of the homogenates and supernatants after KBr treatment of biopsy samples were determined with bicinchoninic acid protein assay reagent (Pierce). The protein concentrations of the purified enzyme were determined by amino acid analysis using norleucine as an internal standard.

Results

Purification of human ileal mucosa PLA_2

Results of purification were summarized in Table I. On reverse phase HPLC, the PLA_2 was eluted as a single peak with 26% acetonitrile (Fig. 1), and the overall recovery of the enzyme activity was 13.6%. The enzyme was eluted at the same position as group II PLA_2 purified from rheumatoid arthritic synovial fluid under the conditions employed (data not shown). Purified PLA_2 migrated as a single band to the same position as human synovial fluid group II PLA_2 on SDS-polyacrylamide gel (Fig. 2), and its apparent molecular weight was estimated to be 14000.

TABLE I

Purification of human ileal mucosa PLA_2

Step	Total activity (μmol/min)	Yield	Specific activity (μmol/min per mg prot.)	Purification (-fold)
Homogenate	38.3	100	0.068	1
KBr extract	35.2	91.8	0.18	2.6
S-Sepharose-1	30.3	79.1	1.31	19.2
Gel filtration	18.8	49.1	5.91	86.9
S-Sepharose-2	20.2	52.7	38.0	559
HPLC	5.2	13.6	306	4500

prot., protein

Using phospholipids containing various head groups (POPG, POPE, POPC), the substrate preference of PLA_2 purified from human ileal mucosa was examined. Ratios of the enzyme activity towards mixed micelles of the phospholipids (0.8 mM) and various concentrations of sodium cholate or deoxycholate (0, 2.5, 5 mM) to that towards sonicated dispersions of POPE are shown in Table II. Like porcine ileal [26] and rat splenic [4] group II PLA_2, the human ileal PLA_2 preferred anionic phosphatidylglycerol or phospholipids in the mixed micelles with anionic bile salts.

Amino acid and NH_2-terminal amino acid sequence analyses of human ileal mucosa PLA_2

The amino acid composition of human ileal PLA_2 was very similar to that of human splenic PLA_2 and

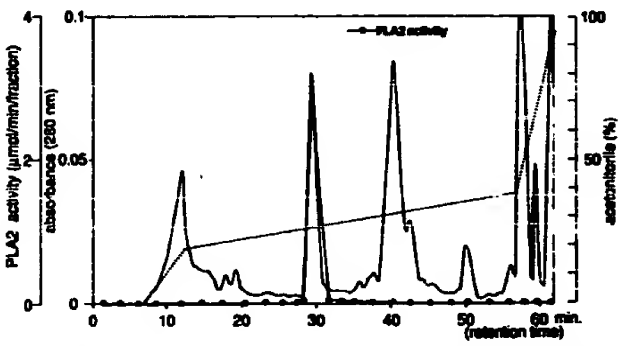

Fig. 1. Elution profile of protein and PLA_2 on a Cosmosil 5C8-300 column. The pooled fractions from a Cellulofine GCL 300-m column were concentrated on a small column (10×5 mm) of S-Sepharose, and then an aliquot of the concentrate was applied to a Cosmosil 5C8-300 reverse phase HPLC column at a flow rate of 0.1 ml/min. The column was developed as described in Materials and Methods.

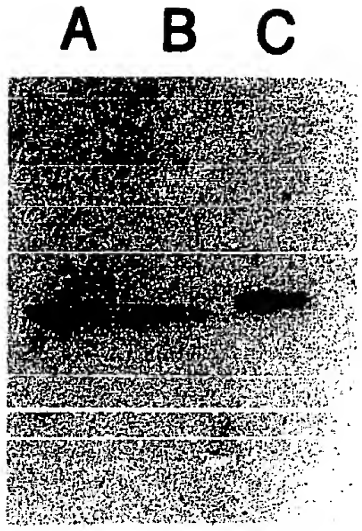

Fig. 2. SDS-polyacrylamide gel electrophoresis of human synovial fluid group II PLA_2 (lane A), human ileal mucosa PLA_2 (lane B), and human pancreatic PLA_2 (lane C). The ileal mucosa PLA_2 migrated to the same position as the synovial fluid group II PLA_2.

showed a high ratio (1.59) of basic to acidic residues characteristic of group II PLA_2s (Table III). The sequence of the NH_2-terminal 55 residues of human ileal PLA_2, NLVNFHRMIKLTTGKEAALSYGFYG-CHCGVGGRGSPKDATDRCCVTHDXCYKXLE, was identical to that of human group II PLA_2 purified from spleen [27], except for a few unidentified residues indicated as X.

Distribution of group II PLA_2 in human ileal and colonic mucosas

Since we previously reported raised PLA_2 activity with a concomitant increase in group II PLA_2 protein level in sera of patients with active Crohn's disease and ulcerative colitis [17,18], we examined the distribution of group II PLA_2 in human lower intestinal tract.

The results of immunoblot analysis showed that the surgical specimens of ileal and cecal mucosas (PLA_2 specific activity of 60.5 and 7.8 nmol/min per mg protein, respectively) contained PLA_2 immunocrossreactive with anti-group II PLA_2 antibody (Fig. 3), but not with anti-pancreatic PLA_2 antibody (data not

TABLE II

Substrate preference of human ileal mucosa PLA_2

The reaction mixture consisted of 100 mM Tris-HCl (pH 8.5), 0.8 mM substrates, various concentrations of detergent, and 5 mM $CaCl_2$. The values are given as ratios of specific activity to that towards POPE dispersions.

Substrate (0.8 mM)	None	Detergent			
		sodium cholate		sodium deoxycholate	
		2.5 mM	5.0 mM	2.5 mM	5.0 mM
POPE	1	20	200	200	80
POPG	900	1200	1200	1200	600
POPC	N.D. [a]	2	40	30	10

[a] N.D., not detectable.

TABLE III

Amino acid composition of human ileal PLA_2

The values in parentheses are the nearest integers.

Amino acids	Human ileum	Human [a] spleen (mol/mol protein)	Human [a] pancreas
Asx	11.2 (11)	11	18
Glx	6.8 (7)	6	8
Cys (Cm)	13.6 (14)	14	14
Ser	9.7 (10)	10	11
Gly	11.6 (12)	11	7
His	4.1 (4)	4	3
Thr	8.9 (9)	10	6
Ala	8.0 (8)	8	8
Arg	9.7 (10)	10	2
Pro	2.4 (2)	2	5
Tyr	7.0 (7)	8	9
Val	3.3 (3)	3	3
Met	1.0 (1)	1	1
Ile	2.1 (2)	2	5
Leu	5.9 (6)	6	7
Phe	5.3 (5)	5	5
Lys	12.7 (13)	13	13
Trp	N.D. [b]	0	1

[a] The amino acid compositions of human splenic [27] and human pancreatic [32] PLA_2s were calculated from the respective sequence data.

[b] N.D., not determined.

Fig. 3. Immunoblot analysis of normal ileal and cecal mucosa homogenates with anti-human synovial fluid PLA_2 antibody. The mucosas were surgically obtained from a patient with Crohn's disease. Each sample contains 10 μg of protein. Lane A, human group II PLA_2 purified from ileal mucosa; lane B, human pancreatic PLA_2 purified from pancreatic juice; lane C, ileal mucosa; lane D, cecal mucosa.

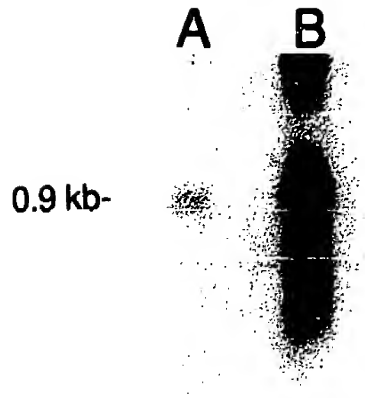

Fig. 4. Northern blot analysis of the histologically normal ileal and cecal mucosas surgically obtained from a patient with Crohn's disease. Each sample contains 10 μg of total RNA. Northern blots were probed with human ileal PLA_2 cDNA. Group II PLA_2 mRNA was observed as a single 0.9 kb band, and the band in lane B was found to be much more intense than that in lane A. Lane A, cecal mucosa; lane B, ileal mucosa.

shown). Each lane gave a band having the same mobility as the purified ileal mucosa group II PLA_2, and the band in the ileal mucosa lane was found to be more intense than that in the cecal one.

Levels of group II PLA_2 mRNA was examined in the ileal and cecal mucosa. On Northern blot analysis, the group II PLA_2 message was observed as 0.9 kb bands both in the ileal and cecal mucosa lanes (Fig. 4), and the length of the mRNA was consistent with that predicted from group II PLA_2 cDNA sequence [6]. The band observed in the ileal mucosa lane was much more intense than that observed in the cecal one, being apparently consistent with their group II PLA_2 protein contents.

PLA_2 activity and IR-PLA_2 II contents in normal terminal ileal and colonic mucosa biopsies obtained from various colon segments of 12 patients undergoing colonoscopy were summarized in Table IV. The latter was determined by a radioimmunoassay after being solubilized by 1 M KBr treatment. Recovery of PLA_2 activity in the supernatant fractions of the homogenates of the biopsy specimen after KBr treatment was $93.8 \pm 5.4\%$ (mean $\pm$ S.E.). Both PLA_2 activity and IR-PLA_2 II contents in terminal ileum were much higher than those in colonic mucosas, and no obvious differences in PLA_2 activity and IR-PLA_2 II contents were found among the various segments of the colon.

TABLE IV

PLA_2 specific activity and immunoreactive group II PLA_2 contents of human terminal ileum and the various segments of the colon

The values are represented as mean and those in parentheses are a range of measured specific activity values.

Portion	PLA_2 activity (nmol/min per mg protein)	IR-PLA_2 II (ng/mg protein)
Terminal ileum ($n = 3$)	50.9 (40.8–61.8)	185.9 (101.7–289.9)
Cecum ($n = 3$)	7.1 (6.3– 8.2)	16.3 (9.0– 28.8)
Ascending colon ($n = 3$)	7.2 (4.7– 8.9)	13.2 (6.1– 17.0)
Transverse colon ($n = 3$)	7.8 (6.1–11.1)	11.8 (5.9– 17.3)
Descending colon ($n = 3$)	9.2 (3.6–13.3)	9.7 (4.3– 16.9)
Sigmoid colon ($n = 3$)	6.2 (4.4– 8.0)	14.1 (4.1– 31.2)

Discussion

In the present study, we have purified a PLA_2 from human ileal mucosa, and determined its NH_2-terminal amino acid sequence and amino acid composition. This 14-kDa enzyme lacked Cys-11, and contained absolutely conserved active site residues, His-48, Tyr-52, and the residues related to calcium-binding to the enzyme, including Asp-49 and the segment from Tyr-25 to Gly-35. Its amino acid composition exhibited high contents of basic residues. Hence, this enzyme is thought to belong to group II enzyme, as PLA_2s in pig ileal mucosa [26], and rat small and large intestinal mucosa [22]. Human ileal mucosa PLA_2 was identical to the group II PLA_2 purified from synovial fluid [6] and spleen [27] as regards its NH_2-terminal amino acid sequence and molecular weight. These results suggest that the ileal PLA_2 is identical to human group II PLA_2 purified from synovial fluid, platelets [6], and spleen [27]. This has been confirmed by the nucleotide sequence determination of the ileal PLA_2 cDNA (Ishizaki, J. and Teraoka, H., personal communication). The sequence was completely identical to that of mRNA cloned from synovial fluid cells [28].

We reported that two forms of IR-PLA_2 II, which were separable by reverse phase HPLC, are present in sera of the patients with inflammatory bowel disease [17]. However, a form eluted later on reverse phase HPLC was not detectable during the purification from the ileal mucosa. The appearance of this form in sera was not specific to inflammatory response, because the similar elution behavior on HPLC was observed on PLA_2 activity in sera of healthy subjects. Its origin and molecular properties should be further evaluated compared with those of PLA_2 purified in this study.

An immunohistochemical study using anti-group II PLA_2 antibody showed that the immunoreactivity with the antibody was detected in human ileum and ascending colon, but not in transverse, descending or sigmoid colon [29]. We previously detected group II PLA_2 immunoreactivity in human descending colon by immunoblot analysis [17]. In this study, we found a rather uniform distribution of the immunoreactivity in the colonic mucosas from cecum to sigmoid colon. It is unclear whether or not this discrepancy was simply due to the difference in sensitivity of detection methods used.

The physiological role of the PLA_2 purified from human ileal mucosa is unknown. The results of this

study on substrate preference showed that the ileal mucosa PLA_2 hydrolyzed POPG more preferably than POPC or POPE, and that POPC was a very poor substrate. This substrate specificity was the same as that of rat and porcine group II PLA_2s [4,26]. In rat small intestine, group II PLA_2 immunoreactivity was rather strongly detected in Paneth cell [30]. Paneth cells contain secretory granules including antibacterial materials, such as lysozyme, IgA and defensin, and thereby thought to be involved in controlling the bacterial milieu of the small intestine. In this connection, the ileal mucosa PLA_2 may play a role in hydrolysis of bacterial phosphatidylglycerol-rich phospholipids and in mediating intestinal inflammation. Further studies, including evaluation of physiological and pathological stimulants for group II PLA_2 secretion from intestinal mucosal cells, are needed for a more precise understanding of the role played by the intestinal PLA_2.

In the previous studies [17,18], we reported the elevation of PLA_2 activity and IR-PLA_2 II levels in sera of patients with active Crohn's disease and ulcerative colitis. Group II PLA_2 was proposed to be an acute phase protein, expression of which is mediated by cytokines [31]. However, the origin of serum PLA_2 in these patients is still unknown. The results of the present study suggested that increased group II PLA_2 in sera of these patients may be, at least in part, caused by its leakage from the ileal and colonic mucosa damaged by inflammation. The more the PLA_2 content is at an inflamed site, the more the enzyme tends to be leaked out to the bloodstream. In Crohn's disease, the diseased area can be distributed into any portions of the gastrointestinal tract. We preliminarily compared the IR-PLA_2 II levels in sera of patients with colonic and small intestinal Crohn's disease; we found no significant difference between the levels [18], although group II PLA_2 was more abundant in the ileal mucosa than in the colonic mucosa. This was not necessarily consistent with the possibility as mentioned above. This point should be addressed by studies on larger populations of patients with Crohn's disease.

Acknowledgments

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